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GIANT SMOOTH MUSCLE FIBERS OF THE CTENOPHORE *MNEMIOPSIS LEYDII:* ULTRASTRUCTURAL STUDY OF *IN SITU* AND ISOLATED CELLS

MARI-LUZ HERNANDEZ-NICAISE, GHISLAIN NICAISE, AND LUC MALAVAL

Histologie et Biologie Tissulaire, Université Claude Bernard, 43 Boulevard du 11 Novembre, 69622—Villeurbanne, France[1]

ABSTRACT

The lobate ctenophore *Mnemiopsis leydii* possesses giant smooth muscle fibers grouped in two sagittal bundles. Functional isolated cells were obtained by an enzymatic digestion of mesoglea and epithelia.

Each bundle is made of 30 to 50 multinucleated cylindrical cells which may reach 35 μm in diameter and 4 cm in length. The nuclei and non-contractile organelles (mitochondria, golgi, rough endoplasmic reticulum) are contained in a discontinuous axial core, surrounded by a thick sheath of myofilaments. Thin (actin) filaments, 5.9 nm in diameter, form irregular rosettes around the thick (myosin) filaments, 16.1 nm in diameter. An actin:myosin filament ratio of 7:2 and a myosin density of 249 filaments per μm^2 were found in cross-sections of relaxed *in situ* cells. No dense bodies nor attachment plates were observed. From the coiled shape of contracted single cells and from the rearrangement of organelles in such coiled cells, we propose that myofilaments are organized in thin long myofibrils attached to the cell membrane at both ends, and that the attachment sites follow two (sets of) enantiomorphic helices. The sarcoplasmic reticulum is a longitudinally oriented 3-dimensional network of narrow tubules among the myofilaments. Its relative volume, estimated from cross sections, amounts to 0.9% of the contractile cytoplasm. No peripheral couplings have been observed, nor any tubular or vesicular invagination of the sarcolemma.

INTRODUCTION

The first giant smooth muscle fiber so far known has been described in a planktonic invertebrate, the ctenophore *Beroe* (Hernandez-Nicaise and Amsellem, 1980; Hernandez-Nicaise *et al.*, 1980). These multinucleated cells can reach several centimeters in length and 50 μm in diameter; they have no striation, dense bodies or attachment plates, at least when observed after conventional processing for electron microscopy.

A simple technique using hyaluronidase and trypsin has been performed to isolate functional muscle cells of *Beroe.* Their ultrastructure and the electrical properties of their membrane are similar to those of *in situ* fibers (Hernandez-Nicaise *et al.*, 1982). Such a single giant smooth muscle fiber provides a multi-purpose model for cell biological studies of smooth muscle. Recent studies using a single cell preparation from a lower vertebrate (Bagby *et al.*, 1971) have already lead to a better understanding of smooth muscle cell organization and function (Fay, 1976; Walsh and Singer, 1981; Fay *et al.*, 1983).

These possibilities are hindered by the limited availability of *Beroe.* To our knowledge, this species is available along the French Mediterranean coast (Station Zoologique at Villefranche-sur-Mer) only from March to May, and as the animals feed upon

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[1] Present address: Cytologie expérimentale, Université de Nice, Parc Valrose, 06034 Nice, France.

other fragile planktonic species they cannot be kept in good condition for more than one or two weeks in the laboratory. We thus tried to identify another species which could (1) yield giant smooth muscle fibers, (2) be available over long periods at low cost, and (3) be kept in a marine laboratory without excessive care.

We found that the lobate species *Mnemiopsis leydii* (Fig. 1) meets all these requirements: it is possible to isolate functional giant smooth muscle fibers from the two sagittal muscle bundles; it is an ubiquitous neritic species of the southern shores of North America and is, for example, common throughout the summer in Woods Hole, Massachusetts; and the animals can be kept several days in still sea water, renewed every day. The possibility of rearing closely related species through long periods has been demonstrated (Baker and Reeve, 1974; Ward, 1974).

The present report describes the method used to obtain functional single muscle cells and gives a detailed electron microscopic description of the *in situ* and isolated cell together with a stereological analysis of the sarcoplasmic reticulum (SR). The ultrastructural features of *Mnemiopsis* giant smooth muscle cells are compared with those of *Beroe*.

A preliminary electro-physiological investigation has been conducted prior to this morphological study to ascertain the physiological integrity of freshly isolated cells

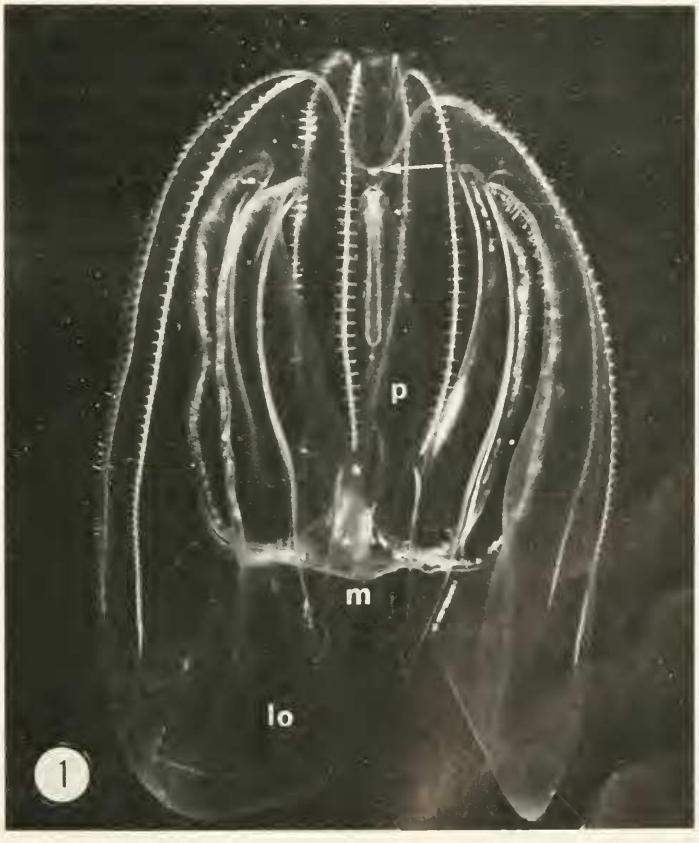


FIGURE 1. Photograph of a living *Mnemiopsis leydii*, viewed from the tentacular side. The animal is swimming downward with mouth (m) and oral lobes (lo) below and aboral organ (arrow) uppermost. The pharynx (p) is visible through the transparent mesoglea. 1.5×.

(Hernandez-Nicaise *et al.*, 1981) and has shown that the resting potentials (average: −56 mV), membrane impedances, and action potentials of isolated and *in situ* fibers do not differ significantly. Further electrophysiological studies are in progress (Anderson, in press).

Materials and Methods

Specimens of *Mnemiopsis leydii* were collected with plastic beakers along the jetty of the N. M. Fisheries Service, Woods Hole, Massachusetts. The two sagittal muscle bundles (Fig. 2) were mechanically removed from the animals. Care was taken to eliminate as much mesoglea as possible, and to preserve the attachment of the muscle cells at both ends (endodermic lining of aboral canal and epidermis of the lips). The dissected bundles were kept in cooled artificial sea water (ASW) prepared according to Cavanaugh (1956) (NaCl, 423 m*M;* KCl, 9 m*M;* $CaCl_2$, 9 m*M;* $MgCl_2$, 23 m*M;* $MgSO_4$, 26 m*M;* $NaHCO_3$, 2 m*M*).

Cell preparation

The procedure devised for *Beroe* muscles (Hernandez-Nicaise *et al.*, 1982) appeared poorly adapted for *Mnemiopsis* muscular bundles, and was modified as follows: after a brief rinse in Ca-free ASW, the dissected bundles were pre-incubated at 30°C for 75–90 min in 0.3% hyaluronidase (type III, Sigma) in nominally Ca-free ASW. After this incubation the mesoglea surrounding the muscle cells was considerably softened and could be removed by dissection.

The tissues were then incubated in 0.05% trypsin (type III, Sigma) and 0.3% hyaluronidase at 30°C for 20–30 min in Ca-free ASW. The bundles were then transferred to Ca-free ASW. They were gently agitated by blowing them in and out of a large bore siliconized Pasteur pipette, until the individual cells were freed from the bundle. The released muscle cells were transferred to cold Ca-containing ASW. From this stage on, siliconized glassware was used.

Electron microscopy

Freshly dissected bundles could not be fixed by immersion into a fixative. Observation of the bundles under the microscope during the onset of such fixation showed that the contact of the extremities of muscle fibers with the fixative triggers such a violent contraction that the muscles break into small fragments and disintegrate.

To prevent this contraction two alternative protocols were devised: (1) the dissected bundles were incubated for 30 min in 0.3% hyaluronidase in Ca-free ASW prior to fixation; the cells remained relaxed when immersed in fixative, or (2) the bundles were incubated for one hour in Ca-free ASW prior to fixation; in this case the cells had an undulated appearance probably due to an interaction between the remaining intact mesoglea and the fixative. All the figures of *in situ* fibers presented in this study were obtained from bundles pretreated with hyaluronidase.

Single cells were transferred by pipette into fixative without any damage.

Bundles and single cells were fixed with 5% glutaraldehyde in cacodylate-buffered ASW (pH 7.7) at room temperature. Following a brief rinse in this buffered isotonic saline, the cells or the whole bundles were post-fixed in 2% osmium tetroxide in the same saline. Some specimens were block-stained with 1% tannic acid (Mallinckrodt) in 0.1 *M* sodium cacodylate (pH 6.5). The specimens were subsequently dehydrated in a graded series of ethyl alcohol followed by three changes in propylene oxide and embedded in durcupan. Silver grey sections were stained with uranyl acetate followed by lead citrate.

Morphometry

Six bundles from different individuals were cut transversally. An electron micrograph at an initial magnification of 10,000× was taken from sections of seven fibers randomly chosen in each bundle. The magnification of the electron microscope was repeatedly calibrated against an optical grating replica (1260 lines/mm). Measurements were performed on prints at an exact magnification of 30,000×, with an image analyzer Videoplan (Kontron, Germany). The total surface of each muscle fiber section and the surface occupied by the axial column and the mitochondria were measured. The difference between these two areas was defined as the contractile cytoplasm area ("Acc").

The area, "Asr," and membrane length, "Bsr," of sarcoplasmic reticulum (SR) were also measured and the number of SR profiles, "Nsr," was counted.

Our hypothesis was that the morphology of the SR (see Results) is consistent with a stereological model of continuous tubules parallel to the main axis of the fiber, as defined by Weibel (1972) for the SR of striated muscle fibers. Under these conditions, the relative area, Asr/Acc, and the relative membrane length, Bsr/Acc, of the SR on a given section are representative of the relative volume, Vv; and if the section is transverse, they are representative of the relative surface, Sv, of the SR in the contractile cytoplasm (Weibel, 1972). These stereological parameters were estimated, after a preliminary statistical study, by averaging the calculated ratios of each section (estimator 1 of case II in Cruz-Orive, 1980), thus: $Vv = \frac{1}{n} \Sigma \frac{Asr}{Acc}$, $Sv = \frac{1}{n} \Sigma \frac{Bsr}{Acc}$, n being the number of sections of measured fibers. In the case of Sv, the value obtained by assuming an isotropic organization $Sv = \frac{4}{\pi n} \Sigma \frac{Bsr}{Acc}$, has also been calculated. The numerical density of the SR tubules in sections was estimated in the same way by $NA = \frac{1}{n} \Sigma \frac{Nsr}{Acc}$.

Quantitative study of myofilaments

The densities and diameters of myofilaments were estimated from micrographs taken from the same sample as above. Measurements were performed on prints at a final magnification of 100,000×.

The number of myosin filaments per μm^2 was counted on cross sections of 14 cells from 4 bundles. A one way variance analysis showed that there was no significant differences between two fields within a same section, between two cells of the same bundle, nor between two cells of different animals.

Therefore a smaller sample was used to determine actin density (4 cells) and filaments diameters (4 cells, 50 filaments of each category per cell).

Freeze-fracturing

For freeze-fracturing studies whole bundles were fixed at Woods Hole in 5% glutaraldehyde in cacodylate buffered, Ca-free ASW. They were kept in ice-cold fixative for several days (3 to 7) until they were processed in our laboratory in France. The tissue samples were thoroughly rinsed in several baths of buffered saline and dissected into small pieces. The specimens were infiltrated with 30% glycerol in the same buffer for 3 hours. Tissue blocks were then mounted between two copper discs, rapidly frozen at −210°C in nitrogen slush, and placed into the double-fracture device of a "Reichert Jung CF 250" unit. They were then fractured at a stage temperature of

−150°C, under a vacuum of 1.10^{-8} Torr without etching. The exposed surfaces were shadowed with carbon/platinum at an angle of 45°. Replicas were cleaned with sodium hypochlorite, repeatedly rinsed in distilled water, and mounted on copper grids.

RESULTS

Light microscope observations on living bundles

The mesoglea of *Mnemiopsis leydii* is crossed by numerous muscle fibers. Most of them are too widely separated from each other and too small in diameter to be interesting either for *in situ* studies or as single cell preparations as is the case for *Beroe* muscle cells. Two sagittal bundles of thick long muscle fibers are known to occur in *Mnemiopsis* (Fig. 2) as well as in other lobates (Chun, 1880), but have been paid little attention; they have even been reinterpretated as connective tissue. From our own observations on living animals, we are now able to give a more precise description of these muscular tracts.

Each bundle consists of 30 to 50 muscle fibers, tightly packed into a flat ribbon contained in the sagittal plane; the bundles follow one edge of the pharynx wall along its entire length. At their aboral extremities, all the muscle fibers in each bundle are attached to the walls of the axial aboral canal which is a continuation of the pharynx cavity immediately below the apical sensory organ (see Hyman, 1940). Muscle fibers

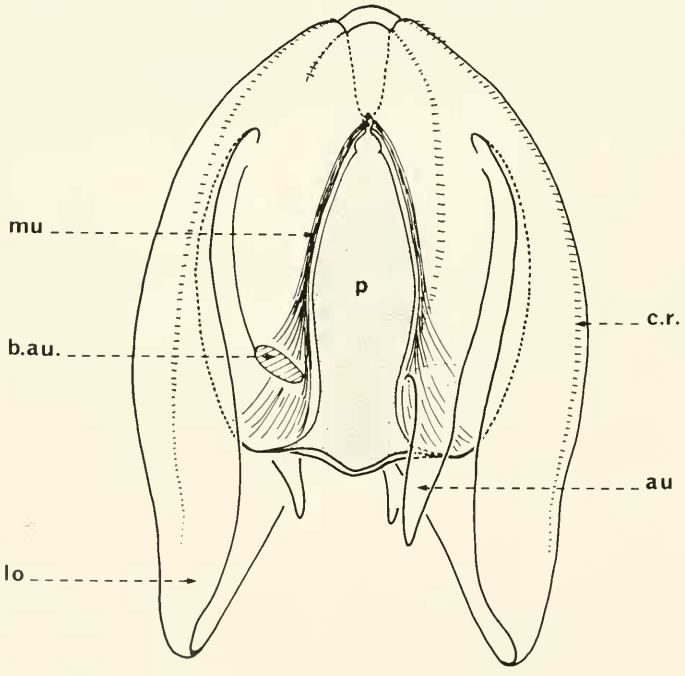


FIGURE 2. Diagram of *Mnemiopsis* drawn after Figure 1 showing the course of the sagittal muscle bundles (mu) along the pharynx (dotted). At their oral extremities, the muscle fibers may either enter the base of the auricle (au) or lips; same legends as in Figure 1. On the left part of the drawing the auricle has been cut away (b.au; cut base of the auricle), and the paratransversal comb row is not represented.

progressively spread out from the bundle and insert on the body wall at various levels from the bases of the auricles down to the lips (Fig. 2).

Each muscle fiber appears as a transparent cylinder running through the entire length of the bundle. The length of the bundle—and thus of the fibers—is directly related to the size (and age) of the animal. The longest fibers are also the thickest. Our electron microscope studies were conducted on a sample of bundles ranging from 1 to 2.5 cm in length, whereas in experiments and fixations on single cells we used the longest bundles, up to 3.5–4 cm.

Light microscope observations on living cells

Undisturbed isolated cells, or cells kept in Ca-free ASW, appear as smooth surfaced cylinders, as in the intact animal. In our experiments, the extremities of single cells either taper into thread-like endings, or swell into a bulbous stump suggestive of resealing which has been demonstrated in *Beroe* muscle cells (Hernandez-Nicaise *et al.*, 1980) (Figs. 3, 4).

Upon weak mechanical or electrical stimulation, the cells contract and become coiled (Fig. 4). The coiling is either clockwise, counter-clockwise, or both, along a given cell. Usually a weak stimulus initiates a localized thickened coil which may spread slowly along the cell, so that part of the whole length of the cell will take this spring-like profile. Such coiled fibers are never observed in an intact animal nor in bundles.

Further stimulation or excess external K^+ (100 m*M*) induces a strong contraction leading to a full shortening of the fiber. Usually it undergoes a brief coiled phase, then shortens dramatically into a thick straight cylinder with a transversally plicated surface (Fig. 5). Such an extreme shortening cannot be obtained *in situ:* in the intact mesoglea a strongly stimulated fiber will break into a row of bead-like fragments. In most cases an isolated cell will not stand such extreme shortening: the membrane usually bursts apart at one end, or the contractile cytoplasm detaches from the cell membrane and recoils at one extremity of the cell.

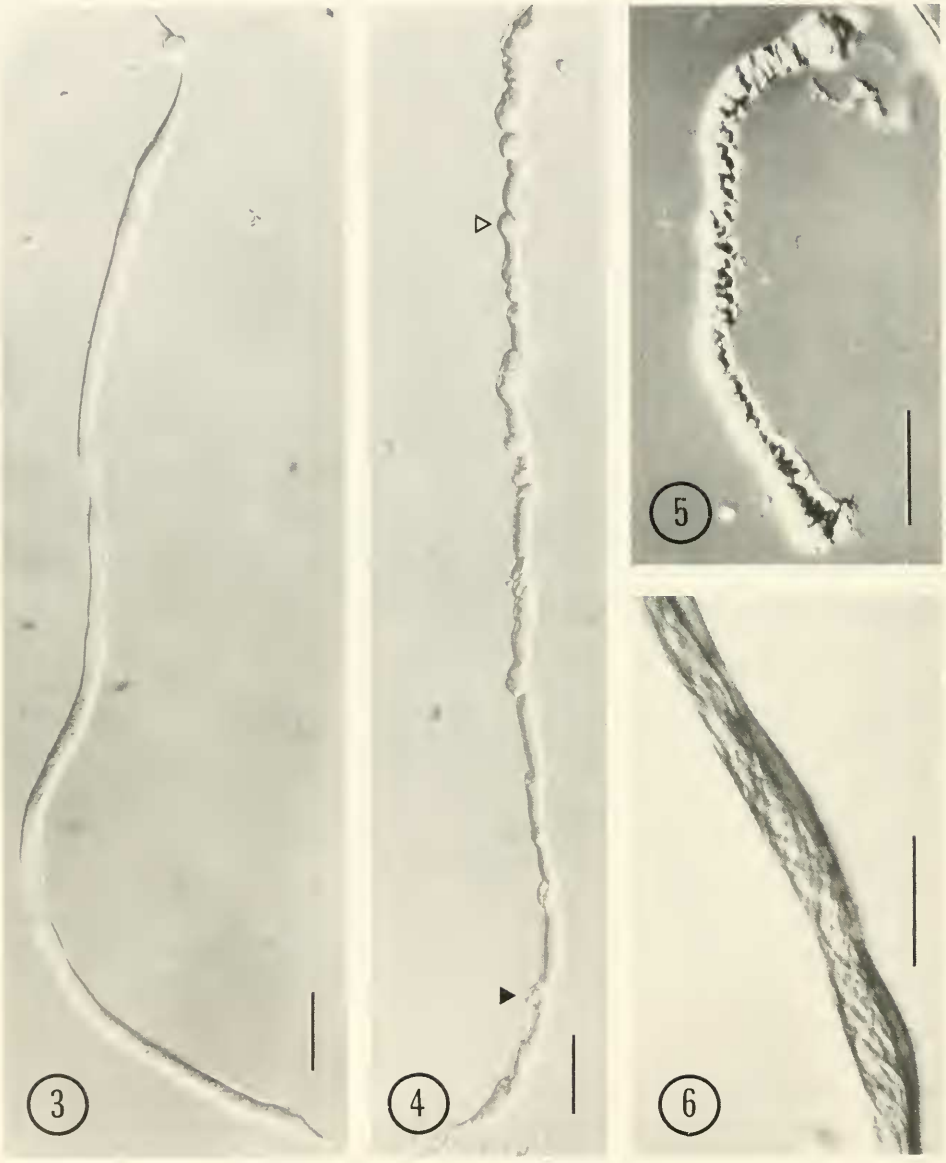
Under Nomarski optics, the fibers exhibit an axial row of nuclei with no intervening septa. The muscle fibers of *Mnemiopsis* thus appear as long multinucleated cells. The contractile cytoplasm shows no transverse or oblique striation. It displays a fibrillar organization: fibrils run along the entire length of the cell and are entwined in crossed helices (Fig. 6).

Electron microscope study

The ultrastructure of cells *in situ* and of single cells appears similar. No modifications suggestive of damage caused by the enzymatic dissociation have been found in isolated cells. The following description is thus largely based on a survey of several bundles, with additional data gained from the study of sections of isolated cells.

Examination of cross sections of various bundles confirms that the diameter of muscle cells is correlated with their length, and shows that the cells of one bundle are fairly homogenous in size. The shortest bundle examined has the smallest fibers, from 3 to 4.3 μm in diameter (average: 3.5 μm, 30 fibers), and the longest bundle has the largest fibers, with diameters up to 36 μm (Figs. 7, 8).

The muscle cells of a bundle are attached to their neighbors by thin strands of dense mesoglea, usually less than 1 μm in width (Fig. 7). Each cell is surrounded by a basal lamina, probably modified by the hyaluronidase treatment, but nevertheless densely stained by tannic acid (Figs. 7, 8, 10).



FIGURES 3–6. Micrographs of living muscle fibers isolated from the longitudinal sagittal bundles of *Mnemiopsis;* Nomarski interference contrast.

FIGURE 3. Relaxed muscle fiber. The upper end has probably been cut during isolation and is slightly bulbous. Note the smooth sarcolemmal surface and the axial row of organelles. Scale bar: 100 μm (100×).

FIGURE 4. Coiled muscle fiber. Parts of the fiber coil clockwise (dark triangle) while others coil counterclockwise (open triangle). Note again the bulbous stump at one extremity. Scale bar: 100 μm (100×).

FIGURE 5. Fully contracted fiber. The cell has the appearance of a tightly compressed spring. The contraction has been obtained by exposing the cell to 100 m*M* external K^+. Scale bar: 100 μm (150×).

FIGURE 6. Relaxed cell, slightly compressed to show the double spiral pattern of fibrils. Scale bar: 50 μm (350×).

FIGURE 7. Part of a sagittal muscle bundle seen in cross section from a small *Mnemiopsis*. The diameter of the cells appears uniform. The cells are mechanically coupled by their longitudinal ridges (r) and/or strands of thickened mesoglea (arrows). Scale bar: 2 μm (7000×).

As observed with light microscopy the axial core contains a row of nuclei. The nuclei are euchromatic with a single large nucleolus, and have a smooth oval outline in relaxed cells (Figs. 7, 8, 11). They are surrounded by various organelles: sacs and tubules of rough and smooth endoplasmic reticulum, small Golgi bodies with their associated vesicles, and mitochondria. Between these organelles the axial cytoplasm may be filled by polysomes and free ribosomes, or by bundles of myofilaments.

Mitochondria are restricted to the central core. Their number increases with the size of the muscle fiber. In our preparations they display a clear matrix which sometimes seems swollen, a feature known to occur in other ctenophores (Hernandez-Nicaise and Amsellem, 1980). If the cells have been allowed to remain in ASW containing Ca^{++} (10 m*M*) the mitochondrial matrix contains several dark granules similar to those described in *Beroe* muscles (Nicaise and Hernandez-Nicaise, 1980).

A peculiar feature of these organelles is the continuity of their external membrane with the outer nuclear envelope. Such contacts have been regularly observed in various sections from different animals.

Myofilaments. In all cells examined, two types of filaments have been found, thick and thin (Figs. 8, 9, 10). Their size and distribution have been studied in cross sections of cells from bundles incubated in Ca-free ASW prior to fixation, these cells are thus presumed to be in a relaxed state.

The thick, myosin-like filaments have a minimum diameter of 15.30 nm ± 1.79 (S.D.). Their density is 249 ± 17 (S.D.) filaments per μm^2 of organelle-free contractile cytoplasm in cross-section, and is remarkably constant in the sample studied, varying within a small range from cell to cell (231–264 filaments/μm^2) and within each cell (less than 10% variation). These filaments are regularly distributed in a nearly hexagonal

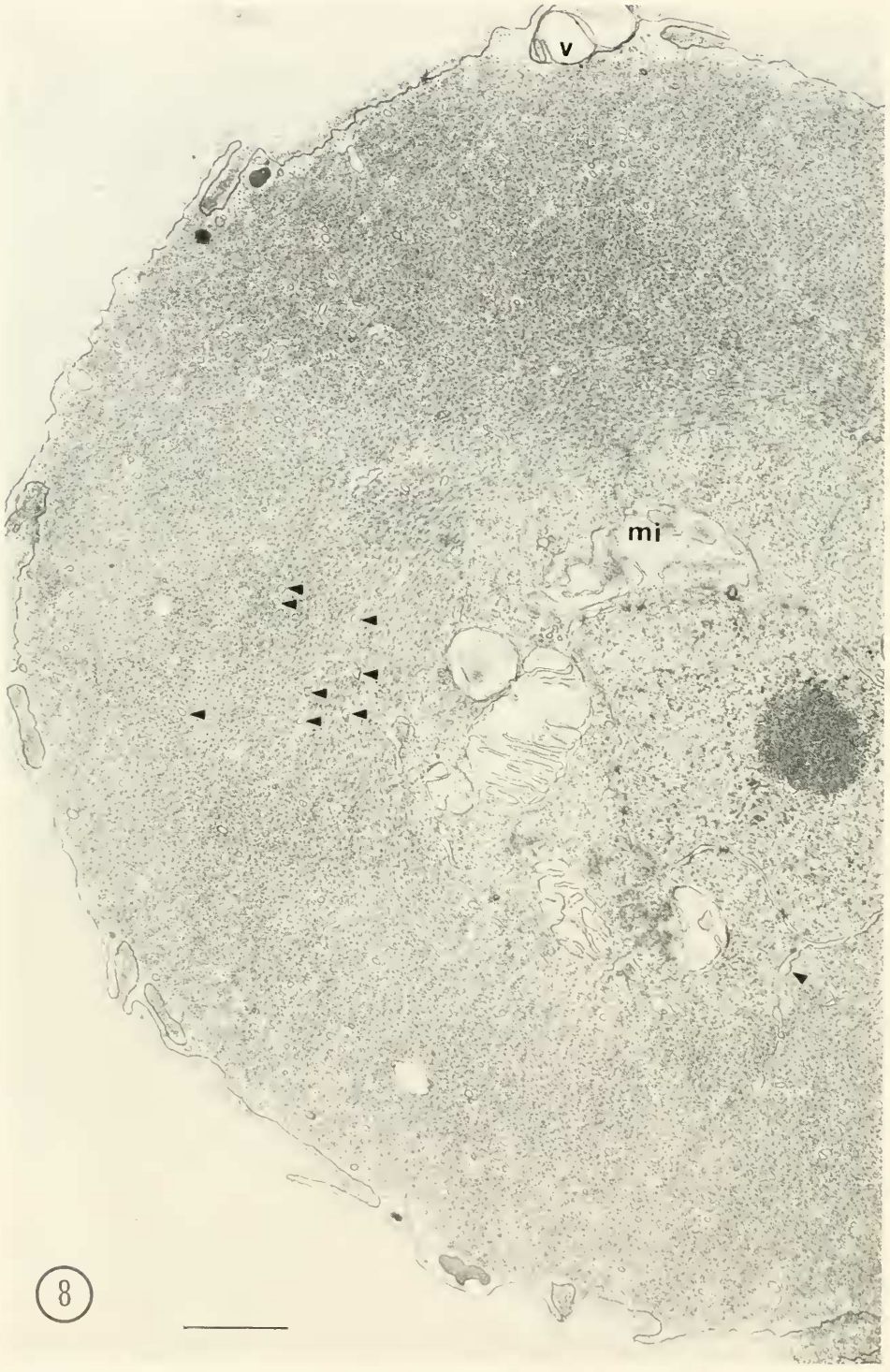


FIGURE 8. Cross section of an *in situ* muscle fiber from a medium sized *Mnemiopsis*. The axial nucleus is surrounded by several mitochondria (mi). The myofilaments are cut at various angles, the overall pattern suggesting an helicoidal arrangement. Note the numerous small SR tubules (arrowheads), nearly all sectioned transversally, and the clear "vesicles" tightly apposed to the cell membrane. Scale bar: 1 μm (14,500×).

pattern (centered hexagons), with a center-to-center spacing varying from 70 to 95 nm (Fig. 9).

The thin, actin-like filaments have a diameter of 5.9 ± 0.84 nm. If the bundles are incubated in a solution containing the S1 fragment of myosin (from chick smooth muscle), the thin filaments exhibit the classical arrowhead configuration. On this basis

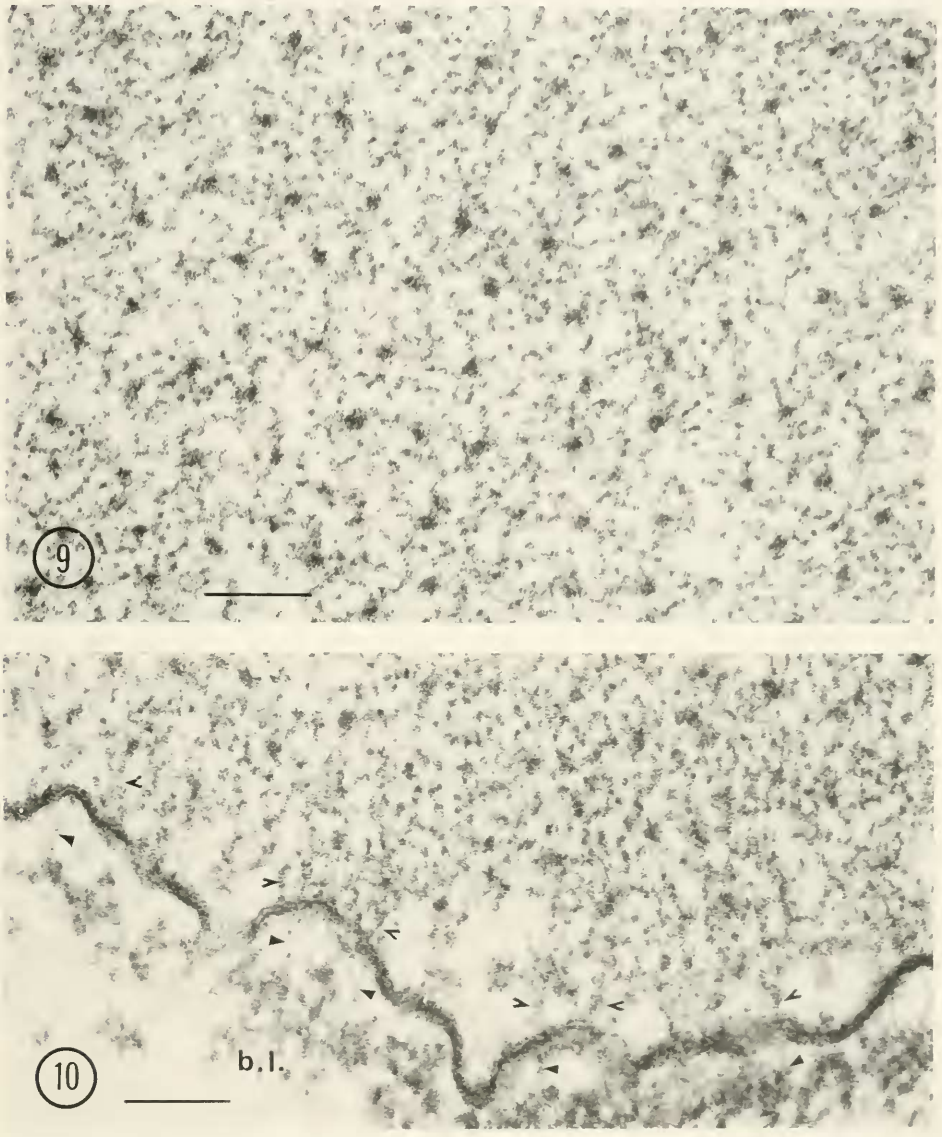


FIGURE 9. Transverse section of thick and thin myofilaments. The thick, myosin-like filaments form a nearly hexagonal lattice. The thin actin filaments form irregular rosettes around the thick myofilaments and are linked by finer microfibrils. Scale bar: 0.1 μm (140,000×).

FIGURE 10. Periphery of a muscle fiber in cross section. Microfibrils link the myofilaments and attach on the plasmalema (open arrows). At the same sites fibrils originating from the basal lamina (b.l.) insert on the external side of the cell membrane (arrowheads). Scale bar: 0.1 μm (140,000×).

they will be referred to here as actin filaments. The ratio of thin-to-thick filaments is very stable, varying from 7.2 to 7.3, with a mean value of 7.22.

In some areas of cross sections the thick and thin filaments are linked by radial cross-bridges stemming from the thick filaments. There is no definite orbital pattern, the thin filaments being arranged in irregular rosettes around the thick filaments (Figs. 9, 10). In restricted patches between thick filaments, adjacent actin filaments are linked by strands of an amorphous electron-dense material building an irregular network.

No striation pattern or dense anchoring structures such as intracytoplasmic dense bodies or peripheral attachment plates have been found in these muscle fibers. However, following tannic acid block staining, discrete patches of fuzzy material can be seen underlying the inner leaflet of the cell membrane. This internal coat is continuous with the material linking the actin filaments. At these same localizations, microfilaments, originating from the basal lamina, attach to the external leaflet of the cell membrane (Fig. 10).

In longitudinal sections of relaxed isolated cells, the myofilaments run nearly parallel to the axis of the fiber and there is no evidence of existing myofibrils. In cross or longitudinal sections of coiled fibers, the overall aspect of the contractile cytoplasm is a zig-zag pattern. The myofilaments appear grouped in bundles cut at various angles, and delineated by a narrow clear space containing a tubule of the SR or one or several microtubules. In a given section the width of such bundles varies from 0.6 to 2 μm (a more accurate estimate requires serial sectioning). We believe that these bundles are the myofibrils observed in living cells.

Surface of muscle cells. Neither cells observed in sections from bundles nor isolated cells exhibit any permanent system of sarcolemmal invaginations. Some occasional infoldings may occur and are easily recognized in tannic acid-contrasted tissues.

In the bundles so far examined, the surface of muscle cells shows longitudinal ridges which appear in cross sections as short (less than 1 μm) finger-like evaginations. The distribution of these ridges very nearly follows an axial symmetry (see Figs. 7, 8).

Single cells do not display these ordered ridges, but instead show numerous slender processes, which are mobile in living cells (Fig. 11).

Sarcoplasmic reticulum. In all the sections observed—from *in situ* and isolated cells—the SR appears distributed among the whole contractile cytoplasm. It is distinctly impregnated by tannic acid in isolated cells which have been fixed immediately upon enzymatic dissociation with no further recovery in normal ASW. From such preparations, the SR appears as a set of longitudinal tubules running along the myofilaments; they may be linked by lateral branches (Figs. 11, 12) or merge into larger vesicles. In longitudinal sections intersecting the nuclei, the tubules are in continuity with the nuclear envelope.

The SR is not particularly abundant in the immediate vicinity of the plasma membrane. No peripheral coupling has been found so far in *Mnemiopsis* fibers, although some vesicles are very close to the cell membrane.

On cross sections the SR profiles are irregularly distributed among the contractile cytoplasm, but their distribution is not random: in most cases the SR sections are aligned on imaginary spirals originating in the axial core toward the periphery of the cell (see Figs. 7, 8).

From our sample of bundles the following quantitative data have been obtained:

The SR profile in cross-section has an average area of 7.6×10^{-3} μm^2 ($\pm 0.3 \times 10^{-3}$ SEM) and an average perimeter of 0.36 μm ($\pm 8.5 \times 10^{-3}$). The SR accounts for 0.91% (± 0.035) of the volume of the contractile cytoplasm, and its relative surface

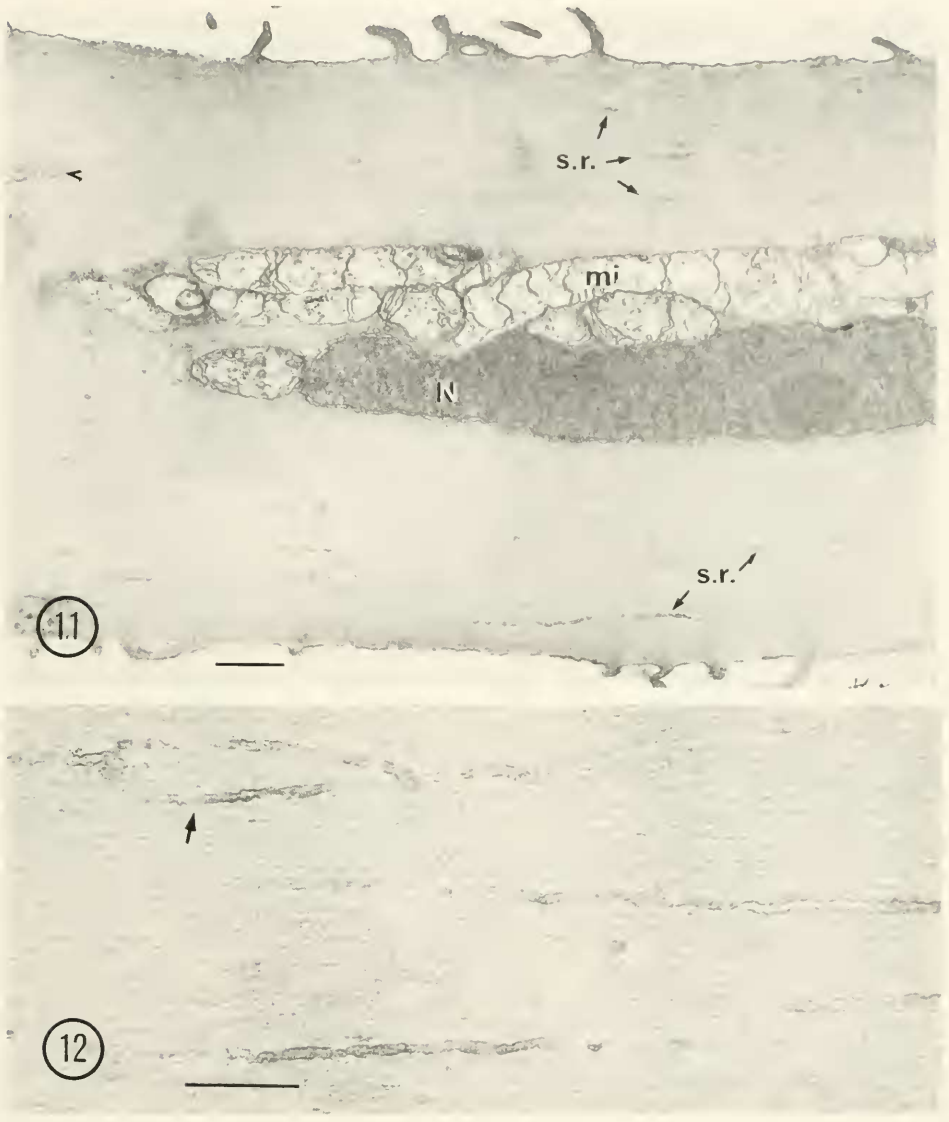


FIGURE 11. Longitudinal section of a relaxed isolated muscle fiber through the axial core. The elongated nucleus (N) is flanked by tightly packed mitochondria (mi). The SR (s.r.) is densely stained by tannic acid. A branching of a tubule is visible at the upper left (open arrow). Scale bar: 1 μm (9000×).

FIGURE 12. Detail of the SR tubules from another section of the same cell as in Figure 11. The arrow points to a "mesh" of the sarcotubular network. Scale bar: 0.5 μm (30,000×).

is 0.45 $\mu m^2/\mu m^3$ ($\pm$0.018) if we assume a complete consistency with Weibel's parallel cylinders model (1972). It may amount to 0.57 $\mu m^2/\mu m^3$ ($\pm$0.023) if we assume that the organization of the SR elements is completely isotropic amongst the myofilaments (see Discussion). The numerical density of SR profiles in the contractile cytoplasm is 1.26 tubules per μm^2 ($\pm$0.061). These values are homogeneous for the whole sample despite the large variability in size of the muscle cells (one way variance analysis, $F = 1.40$).

There is a slight but significant correlation between the value of the ratio Asr/Acc (or Bsr/Acc) and the area of contractile cytoplasm of the section if they are calculated on each section ($r = 0.35$ in both cases, $P < 0.5\%$).

Junctions. We looked for intermuscular junctions in all bundles studied. Few were found with most of them located between fibers of the periphery of the bundles.

At these junctions the plasma membranes are apposed along variable lengths (0.3–4 μm), and may form close contacts at some restricted patches. At these close contacts, tannic acid post osmium stains the intercellular space—4 to 5 nm wide—

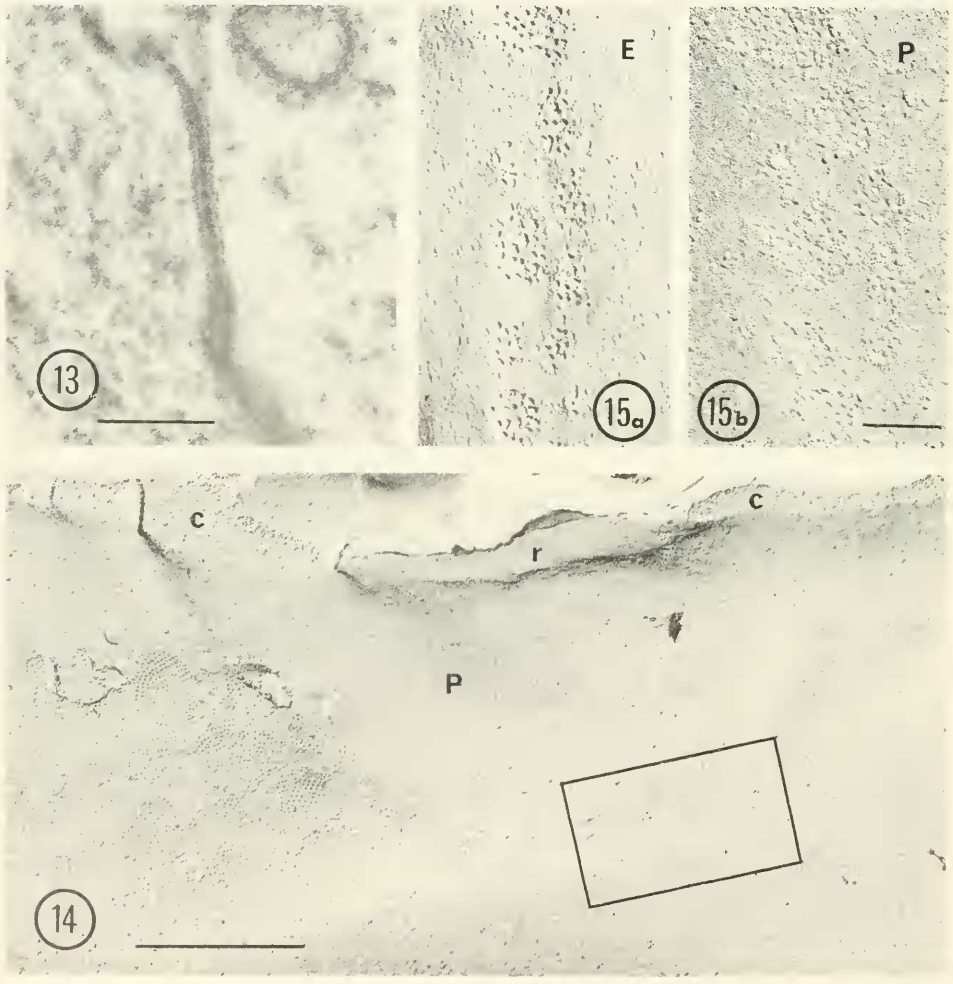


FIGURE 13. Junction between two cells of the bundle. Gap is stained giving rise to a pentalayered structure (tannic acid 1% post osmium). Note the thin bridges across the gap. Scale bar: 0.1 μm (150,000×).

FIGURES 14–15a, b. Freeze-fracture replicas of a muscle fiber.

FIGURE 14. The cleavage plan exposes the P face of the cell membrane and runs through a longitudinal ridge (r) thus exposing a band of cytoplasm (c). The replica shows a large array of polygonal gap junctional plaques, intercalated with particle-free aisles. Scale bar: 0.5 μm (44,000×).

FIGURES 15a, b. Complementary freeze-fracture replicas of part of the junction of Figure 14. E-face particles (Fig. 15a) correspond to a honeycomb array of pits on the P-face (Fig. 15b) (framed area in Fig. 14). Scale bar: 0.1 μm (100,000×).

thus revealing a pentalayered structure (Fig. 13). The intercellular space contains bridges repeating every 16–20 nm.

In freeze-fracture replicas, features characteristic of gap junctions were found on some muscles (Figs. 14, 15a, b). The E-face, or ectoplasmic fracture, of the muscle membrane exhibits polygonal clusters of 11–14 nm particles (Fig. 15a) and a complementary ordered lattice of pits—with a 17 nm center-to-center spacing—on the P, or protoplasmic fracture, face (Figs. 14, 15b). The clusters may be isolated or appear in large arrays (Fig. 14).

In any cross section of a bundle, nearly each muscle profile is in contact with one or two clear circular profiles (0.3–1 μm in diameter) encased between two projections of the muscle membrane. These vesicles do not contain any structure except for an occasional network of microfilaments. At some discrete points the muscle cell and the "clear vesicle" come into close contact, with an intercellular space of 20–40 nm. Survey of the mesoglea surrounding the bundle has not given evidence for a special category of cell as the origin of these structures. We have found that muscle cells of the bundle and of the neighboring mesoglea can emit "blisters" of variable sizes which are apparently empty. We have not observed such evaginations in contact with a neighboring muscle cell. In some cases these "blisters" make reflexive contact with the mother cell. Some of the gap junctions observed in our freeze-fracture replicas may thus fall into this category of junctions.

Discussion

Viability of isolated cells

Our light and electron microscope observations together with the electrophysiological data reported previously (Hernandez-Nicaise *et al.*, 1981) demonstrate that enzymatically isolated muscle cells of *Mnemiopsis leydii* are functional cells: (1) the membrane appears ultrastructurally and physiologically intact, (2) the cells contract if stimulated or in the presence of excess of K^+ ions in the bathing fluid, and (3) the ultrastructural features appear unchanged.

Myofilaments

We consider that the thick and thin filaments are myosin and actin filaments, respectively. As already mentioned the thin filaments are decorated by the S1 subfragment of heavy meromyosin, and preliminary data from gel electrophoresis indicate that *Beroe* muscle cells contain myosin but not paramyosin (A. V. Somlyo, pers. comm.).

In ultrastructural cross sections the overall pattern of actin and myosin is very similar to the distribution observed in *Beroe* muscle cells (Hernandez-Nicaise and Amsellem, 1980). However, the quantitative data for myosin density and actin-to-myosin ratio differ significantly between the two species as reviewed in Table I.

The actin-to-myosin ratio is distinctly lower than the values given for vertebrate smooth muscle (see review in Somlyo, 1980) and is closer to the figures published for several invertebrate phyla (see review in Hernandez-Nicaise and Amsellem, 1980; Plesch, 1977).

Organization of contractile units

One of the most striking features of *Mnemiopsis* muscle cells, apart from their length, is the absence of peripheral and intracytoplasmic dense bodies.

TABLE I

Comparison between morphological parameters of Mnemiopsis leydii[a] *and* Beroe ovata[b] *muscle filaments*

	Mnemiopsis leydii in situ cells relaxed	*Beroe ovata in situ* cells state unknown	*Beroe ovata* isolated cells relaxed	*Beroe ovata* isolated cells coiled
Thick filament diameter	15.30 ± 1.79 nm	16.4 ± 0.3 nm	16.11 ± 2.12 nm	16.11 ± 2.12 nm
Thick filament density	249 ± 17 fil./μm^2	320–450 fil./μm^2	457 ± 15 fil./μm^2	350–660 fil./μm^2
Thick filaments spacing	70–95 nm	40–60 nm		
Thin filament diameter	5.9 ± 0.84 nm	6.2 ± 1.6 nm	6.3 ± 0.6 nm	6.3 ± 0.6 nm
Thin/thick filament ratio	7.22	2.5–7.5	5.22 ± 0.21	4.88 ± 0.29

[a] Means ± S.D.

[b] Data from Hernandez-Nicaise and Amsellem (1980) and Hernandez-Nicaise *et al.* (1982).

In vertebrate smooth muscles the dense bodies are the morphological and functional correlates of fragmented Z lines (Bond and Somlyo, 1982). Their spatial distribution in oblique strands across the cell points to an organization of the myofilaments into small contractile units (Bond and Somlyo, 1982; Fay *et al.*, 1983). A similar type of organization is found in invertebrate smooth muscles like the byssus retractor of *Mytilus* (Sobieszek, 1973). From our observations reported in the present paper, we propose that, as in *Beroe* (Hernandez-Nicaise *et al.*, 1982), the myofilaments may be organized into two sets of thin myofibrils attached at their extremities on the muscle membrane along two (or two sets of) enantiomorphic helices.

The small patches of sarcolemma coated internally by a filamentous coat are likely candidates as mini-attachment plates. The microfilaments linking those patches to the basal membrane are suggestive of fibronectin microfilaments known in other cell types to "cooperate" with actin bundles (Singer, 1979; and review by Hynes, 1981). Such bonds between the muscle membrane and the mesoglea may explain the fragmentation of muscle fibers under the action of fixatives.

Each of the oblique myofibrils may be constituted of serial units, *i.e.*, pseudosarcomeres. The diameter of the muscle fibers and the low angle between the myofilaments and the fiber axis at rest, call for very long fibrils; but the possibility of a 75% shortening of single fibers implies that a fibril is made of a series of units with an important overlapping of myosin and actin filaments, rather than a single pseudosarcomere with one set of myosin filaments. The staggering of myosin and actin filaments in such small myofibrils accentuated by an unavoidable shearing, may thus account for the fact that in cross sections actin and myosin filaments are distributed as in A-bands of obliquely striated muscles fixed in a contracted state (see review in Rosenbluth, 1972).

The structural and biochemical equivalents of intracytoplasmic dense bodies therefore have to be demonstrated in order to establish the existence of serial pseudosarcomeres. Indications of such attachment structures have already been obtained from electron microscopy of highly stretched *Beroe* muscles (unpub. obs.).

Z elements with little or no contrast have been shown (if not specifically reported) in other primitive metazoans, namely Cnidaria. In figures published by various authors, we have noted that in cnidarian epitheliomuscular cells, the smooth muscle fibrils are devoid of dense bodies, and that striated myofibrils display a thin, wavy strand of fuzzy material in place of Z lines (Chapman, 1974; Amerongen and Peteya, 1976; Keough and Summers, 1976; Anderson and Schwab, 1981). Hoyle (1983) reports a striated muscle in a primitive Crustacean with "barely discernible Z regions."

Mnemiopsis muscles (and all other ctenophore muscles we have observed so far) also lack the so-called intermediate filaments, or desmin filaments. From published figures these filaments are equally absent in Cnidaria muscles. Desmin filaments occur in both smooth and striated muscles, and are associated with dense bodies (Small and Sobieszek, 1977; Bond and Somlyo, 1982) and the Z line (Behrend, 1977; Lazarides, 1980), respectively. The protein α-actinin is one of the main components of the Z line and dense bodies (Ebashi *et al.*, 1966; Schollmayer *et al.*, 1976; Endo and Nasaki, 1982) and is responsible for most of the electron-opacity of these structures together with desmin and vinculin (see review in Lazarides, 1980). Ctenophores and Cnidaria may lack α-actinin and desmin and may use other proteins (see review in Weeds, 1982), with a weak affinity for electron microscope stains, as bonds between the actin filaments and anchoring structures.

Sarcoplasmic reticulum

The SR of the giant muscle cells of *Mnemiopsis* is very similar in its morphology and distribution to that of *Beroe* giant muscle cells (Hernandez-Nicaise and Amsellem, 1980; Malaval *et al.*, 1981; Malaval, 1982). In both species the heterogeneous distribution of SR in the myoplasm and the alignment of SR profiles in cross sections along spirals, suggest an ordered three-dimensional pattern of this organelle along the fiber, which may reflect to some extent the organization of the myofibrils (Hernandez-Nicaise *et al.*, 1982; Malaval, 1982).

Our estimates of SR relative volume and correlated parameters have been calculated on the assumption that the SR distribution in relaxed *Mnemiopsis* muscles follows Weibel's model of parallel tubules (1972). Table II gives the values obtained for *Mnemiopsis* and *Beroe* muscles. It is theoretically possible to assess the relevance of this model for a given specimen by comparing the values obtained for Sv in different planes of section (Eisenberg *et al.*, 1974). However, such a control appears only feasible on striated fibers, for which an estimate of the angle between the plane of section and the axis of the cell can be calculated from the length of sarcomeres. With *Mnemiopsis* fibers we can only consider the extreme values of Sv, the "real" value probably being closer to the figure obtained with Weibel's anisotropic model.

To summarize, the relative volumes of SR in *Mnemiopsis* and *Beroe* muscles are closely similar in value but in *Mnemiopsis* the tubules are smaller and more numerous per cross sectional unit.

TABLE II

Comparison between morphological parameters of SR in muscle fibers of Mnemiopsis leydii[a] *and* Beroe ovata[b]

		Average area of SR tubule in cross section	Average circumference of SR tubule in cross section	Average diameter of SR tubule in cross section	Relative volume of SR in % of contractile cytoplasm	Surface density of SR, expressed in $\mu m^2/\mu m^3$	Numerical density of SR tubules in cross sections
Mnemiopsis		$7.6 \cdot 10^{-3}\ \mu m^2 \pm 0.3 \cdot 10^{-3}$	$0.36\ \mu m \pm 8.5 \cdot 10^{-3}$	$0.114\ \mu m$	$0.91\% \pm 0.035$	0.45 ± 0.018	1.26 ± 0.026
Beroe	1	$15 \cdot 10^{-3}\ \mu m^2 \pm 1 \cdot 10^{-3}$	$0.48\ \mu m \pm 20 \cdot 10^{-3}$	$0.152\ \mu m$	$1.24\% \pm 0.070$	0.40 ± 0.020	0.90 ± 0.070
	2	$19 \cdot 10^{-3}\ \mu m^2 \pm 2 \cdot 10^{-3}$	$0.59\ \mu m \pm 30 \cdot 10^{-3}$	$0.186\ \mu m$	$0.70\% \pm 0.50$	0.22 ± 0.020	0.38 ± 0.030

[a] Means ± S.D.

[b] Data from Malaval (1982). The values were obtained from two samples from two animals and the two sets of figures differed significantly and are thus given separately (see Schmalbruch, 1979, for a similar problem with muscle parameter variability in a vertebrate).

The wide range of sizes in our sample of sagittal bundles of muscle cells in *Mnemiopsis* has enabled us to find a positive correlation between fiber size and the SR volume.

The relative volume of SR of ctenophore giant smooth muscles is thus low if compared with the figures generally given as reference for vertebrate smooth muscle, namely 5.75% in vascular smooth muscle and 2% in phasic smooth muscle (Somlyo, 1980). However very similar figures have been reported for mammalian smooth and striated muscles (Schmalbruch, 1979; McGuffee *et al.*, 1981). Furthermore, these studies show the unsuspected influence in such quantitative studies of various factors extrinsic to the preparation such as the fixation procedure (McGuffee *et al.*, 1981), the buffer used in fixing and rinsing solutions (Moriya and Miyazaki, 1979) and the physiological condition of the animal (Schmalbruch, 1979).

A relative volume of 1% is estimated by Somlyo (1978) as the minimum amount of SR to allow a release of Ca^{++} sufficient for activation of the contractile proteins. Assuming that this statement is true for ctenophores whose internal fluid and temperature differ widely from those of mammals, the value of 0.91% found in *Mnemiopsis* muscle cells is not too low to rule out that this organelle is involved in the onset of contraction.

Another important indication for the role of SR in contraction is the presence of peripheral couplings (or dyads) between sarcoplasmic cisternae and the plasma membrane which presumably are crucial in excitation-contraction coupling. No couplings have been observed in *Mnemiopsis,* and very few cisternae are found close to the membrane. In *Beroe* muscles most of these couplings are found at neuromuscular and intermuscular junctions. We may have missed them in *Mnemiopsis* as we were not specifically looking for neuromuscular junctions. This observation does not necessarily exclude the intervention of the SR in ctenophore muscles in excitation-contraction coupling and certainly does not rule out a role for this organelle in the relaxation of these giant fibers (Malaval *et al.*, 1981; Malaval, 1982).

If we adopt the criteria of classification proposed by Josephson (1975) and Plesch (1977), the giant muscles of *Mnemiopsis* and *Beroe* clearly fall into the same category. The muscle cells of the sagittal bundles of *Mnemiopsis* show fewer cross-bridges available per actin filament and fewer mitochondria than *Beroe* muscles. They are thus likely to be less powerful, less sustained, and slower than *Beroe* muscle cells. This is consistent with the behavioral data (Swanberg, 1974; Harbison *et al.*, 1978).

Junctions

The few intermuscular junctions observed in the sagittal bundles of *Mnemiopsis* present the features of gap junctions, and more precisely the ultrastructural characteristics of E-gap junctions which are predominantly found in invertebrates (see reviews in Flower, 1977; Peracchia, 1981; Larsen, 1983). The precise distribution of these junctions among the muscles of a bundle remains to be evaluated. In vertebrates, gap junctions are widely accepted as the ultrastructural correlate of electrical coupling (Peracchia, 1981). They occur between smooth muscle cells (Fry *et al.*, 1977) which work in a coordinated fashion; for example, in a pregnant uterus the number of gap junctions between myometrial cells increases significantly at the onset of labor (Garfield *et al.*, 1977).

The contacts described in this study may link together the fibers of the sagittal bundles. To date however, an electrical coupling between muscles of a bundle has not been found (Anderson, in press). The empty "blisters" point toward another possibility: they may be broken parts of radial muscle fibers destroyed during dissection. Such a possibility is also suggested by Anderson (in press).

With the isolation technique reported in this study, it is possible to use the giant

smooth muscle fibers of *Mnemiopsis* for a variety of studies while controlling them individually under a simple dissecting microscope. In addition, *Mnemiopsis* sagittal bundle of parallel fibers can be used as multicellular homogeneous sample for quantitative studies.

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